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FEEDING BEHAVIOR IN *HYDRA*. I. EFFECTS OF *ARTEMIA* HOMOGENATE ON NEMATOCYST DISCHARGE*

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Abstract

Inhibition of desmoneme and stenotele nematocyst discharge occurs when *Hydra attenuata* are fed to repletion. Inhibition can be induced by the application of prey homogenates in the external medium. The onset of inhibition is relatively rapid (<30 s) while the release from inhibition is much slower (>20 min). Inhibition is concentration-dependent. Gel chromatography separation of homogenate shows that the inhibitory substance(s) have a molecular weight greater than 5000. These substances cause the strongest stenotele inhibition and are least effective in activating the feeding reflex (mouth opening and tentacle concerts) which is caused by smaller molecular weight substances. The receptor sites for the inhibitory substance(s) are located on the external surface of the hydra tentacle. Accumulation of prey substances may be the mechanism by which stenotele discharge is inhibited when hydra are fed to repletion.

Introduction

Nematocysts are highly specialized organelles that serve several functions in coelenterates. Nematocysts are used to help capture and kill prey. In some coelenterates, nematocysts are also necessary for locomotion and defense (Picken and Skaer, 1966; Mariscal, 1974).

In Hydra two types of nematocysts are involved with the feeding response. Desmonemes function in prey capture and have a tightly wound thread which wraps around the prey. Stenoteles have a penetrant shaft and function as killing nematocysts by piercing the prey and releasing a lethal toxin (Ewer, 1947). When hydra are fed to repletion, however, they lose their ability to discharge nematocysts. Several causes for nematocyst inhibition have been proposed. (1) Hydra become less responsive to stimulation and fewer stenoteles are brought to bear against the prey (Burnett *et al.*, 1961). (2) Either a metabolite from the prey or a product of digestion inhibits nematocyst discharge (Smith *et al.*, 1974). (3) A factor from the nematocysts themselves inhibits nematocyst discharge (Ruch and Cook, 1984).

We now present evidence that the discharge of stenotele nematocysts can be inhibited by the external application of prey substances and that the receptor sites for these substances are located on the tentacle.

Materials and Methods

Specimens of *Hydra attenuata* from a single asexually reproducing clone were used in all experiments (Kass-Simon and Potter, 1971). They were raised in BVC

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solution (Loomis and Lenhoff, 1956), and were fed daily from a culture of *Artemia* nauplii (brine shrimp).

Brine shrimp homogenates were prepared by concentrating large quantities of brine shrimp nauplii in a filter (Whatman #4 paper) and washing them thoroughly with distilled water. The nauplii were drawn off with a syringe and the concentrate was repeatedly forced through the bore of an 18 gauge needle until its consistency was smooth. This crude homogenate was centrifuged in a clinical centrifuge ($\sim 1000 \times g$, 5 min). The water-soluble middle layer was drawn off and recentrifuged to complete the removal of insoluble material. The finished homogenate was then divided into samples of 1 to 3 ml and frozen at −5°C until use.

The relative concentrations of various homogenates were determined by collecting 1 ml subsamples of brine shrimp from the shrimp collected from the mass cultures. These were counted and the number of brine shrimp per ml computed.

Stenotele discharge was defined as being inhibited when a hydra rejected, in succession, five offered brine shrimp that made contact with the tentacles and that were still swimming normally after 30 s (Smith *et al.*, 1974). Brine shrimp were introduced into the medium surrounding the hydra with a pasteur pipet and were directed towards the tentacles with forceps. To be classified as having made contact, brine shrimp must have distinctly moved a tentacle. Slight brushes with the tentacle were difficult to discern and were not counted. Hydra were transferred from dish to dish using large bore dropping pipets.

Brine shrimp killing was grouped into two categories: (1) brine shrimp that were captured and killed, and (2) those that made contact but were not captured or killed. Brine shrimp which were initially captured, but then escaped were classified in group 2.

Feeding hydra to repletion

Hydra were placed in 10 ml of BVC solution in a 60 × 15 mm glass petri dish, and were offered 2 brine shrimp every 5 minutes for 150 min (30 trials). During the experiment the number of brine shrimp captured, killed, and ingested was monitored. If brine shrimp made contact with the tentacles and were not captured, brine shrimp continued to be offered until either two brine shrimp were captured or a total of five offerings were made.

Inhibition of stenotele discharge

Hydra were placed in 5 ml of 1/25 dilutions of brine shrimp homogenates and were tested for inhibition of stenotele discharge as above. Animals which displayed significant inhibition of stenotele discharge were then transferred to fresh BVC solution for 1 min. They were again transferred to fresh BVC solution for 45 min and again tested with 5 brine shrimp. Control animals were placed in 5 ml of BVC solution and tested for stenotele discharge after 5 min.

Effects of homogenate on stenotele discharge

Two sets of experiments were performed to test the effects of homogenate on nematocyst discharge. Hydra tentacles were ablated 24 h prior to testing. They were then placed in 1/25 dilutions (0.74 mg/ml protein) of brine shrimp homogenates. First, individual tentacles were placed on a glass slide in the diluted homogenate for 5 min and covered with a coverslip; the number of discharged nematocysts were counted under 400× magnification. An equal number of control tentacles were

placed in BVC solution. In addition, tentacles in diluted homogenate were placed in a 0.15 × 1.0 cm cell and the number of discharged nematocysts were counted after 5 and 25 min while being observed under an inverted microscope (400×). Control tentacles were placed in BVC solution.

Time course of inhibition and dose response

The onset of inhibition was determined by placing individual hydra in 1/25 dilutions of brine shrimp homogenate. Hydra were transferred into the homogenate and tested with five brine shrimp at given time intervals after transfer.

To test for recovery from inhibition, several hydra were placed in 1/25 dilutions of homogenate for 5 min, rinsed with fresh BVC solution, and then rapidly transferred into separate dishes containing 5 ml of BVC solution. They were then tested with 5 brine shrimp at regular time intervals ranging from 2.5 min to 22.5 min.

To determine the concentration of homogenate required for inhibition, the homogenate was diluted serially by factors of 10, resulting in dilutions of 10^{-1} to 10^{-6}. Individual hydra were placed in 5 ml of diluted homogenate for 5 min and were then tested with 5 brine shrimp. The protein concentration of the homogenate was determined by measuring the protein concentrations of an homogenate from the same batch (Lowry *et al.*, 1951).

Extraction of inhibitory substances

Gel filtration chromatography (Determan, 1969) was used to fractionate the homogenate. A 15.5 by 1.5 cm column with a polystyrene bed support was packed with sephadex G-25-80 beads (Sigma Chemical Co.). Solvent (BVC solution) was placed in a reservoir raised several feet above the column to create a slight positive pressure. A 1-ml sample of homogenate was used for each experimental run. Sample collection began 50 drops prior to the first appearance of homogenate fractions from the column. Fifteen 20-drop samples were collected. The first appearance of homogenate fractions from the column could be clearly observed due to the cloudiness of the fraction and the slight orange color. This correlated closely with calculations of protein elution from Determann (1969).

Each of the 15 fractions was diluted by 1/20 with BVC solution. One hydra was placed in 5 ml of diluted fraction for 5 min. The level of stenotele discharge, mouth opening, and tentacle activity was determined. Numerical values, as given in Table I, were assigned to each of the responses for data analysis. In a separate set of experiments, 5×10^{-4} *M* reduced glutathione (GSH, Sigma) was run through the column to locate the fractions in which substances that activate the feeding response would be found (Loomis, 1955).

Localization of receptors

To test for receptors in the gastrovascular cavity, injections of homogenate were made through the basal pore. The injection apparatus was composed of drawn out polyethylene tubing attached to a Hamilton 10 microliter syringe (Smith *et al.*, 1974).

First, hydra were injected with 1.0 μl of whole homogenate, and tested with 2 brine shrimp every 5 min for 35 min. At the end of 35 min additional homogenate was injected until it could be seen leaking from the hypostome. Two minutes later the hydra were again tested for stenotele discharge using 5 brine shrimp. As a control, 1.5 μl of air was injected. Air bubbles remain intact and can easily be seen in the gut. After 5 min the hydra were tested for stenotele discharge with 5 brine shrimp.

TABLE I

Numerical values assigned to specific aspects of the feeding behavior in hydra: mouth opening and tentacle activity

Mouth opening response:	Numerical value:
Hypostome protruding from tentacle ring. Slight opening.	1
Moderate mouth opening. Mouth clearly open.	2
Large mouth opening, hydra may be inverting itself.	3
Tentacle activity:	
Slight activity, periodic concerts.	1
Continuous concerts.	2
Tentacle writhing.	3
Tight writhing, tentacles may have entered mouth.	4

Partial values given for activities between the defined steps.

In another series of experiments, hypostomes with attached tentacles (ablated 24 h earlier) were placed in 0.25 ml of BVC solution containing 10 μl of homogenate. The animals were allowed 5 min to relax and were tested with 5 brine shrimp. Control animals were placed in BVC solution only. In addition, isolated tentacles were tested by the same procedure.

Different regions of a given tentacle were also tested for inhibition of stenotele discharge. A small quantity of homogenate was applied to the proximal half of a tentacle using a 10 μl syringe. The extent of the cloud of homogenate could clearly be seen. The proximal half of the tentacle was kept in the homogenate for 2 min and then relocated to a clear region of the depression slide. Brine shrimp were added to the culture medium and the number of random contacts or kills were recorded for 2 min for each half of the tentacle.

If the proximal half of the tentacle became inhibited, that portion was ablated from the whole tentacle and placed in fresh BVC solution. It was allowed 1 h to recover and was then retested for stenotele discharge as above.

Statistical analyses were done on the University of Rhode Island computer system with software from Statistical Analysis Systems (Box 800, Cary, North Carolina). Where applicable, Wilcoxon Rank Sums, Kruskal-Wallis K Samples, and Linear Regression tests were used. Paired data were analyzed using the Wilcoxon Signed-Rank Test (Lentner, 1975).

RESULTS

Our observations with respect to hydra's behavior when fed to repletion (Fig. 1) essentially confirm the results of Smith *et al.* (1974) and Ruch and Cook (1984). The hydra readily killed the brine shrimp offered to them (trials 1–10). However, as additional shrimp were added to the hydra, the number of brine shrimp killed decreased. When the killing of brine shrimp began to decrease (trial #11) the hydra had killed 169 out of 170 brine shrimp (>99%). From trial #11 through #30 killing was reduced to <30% (89/320 brine shrimp). Ingestion was also reduced; 92% of the brine shrimp killed in the first 10 trials were ingested, whereas in the latter trials <40% were ingested.

During the latter trials the killing of brine shrimp by individual hydra were quite variable. A given hydra could be completely inhibited for two or more periods and

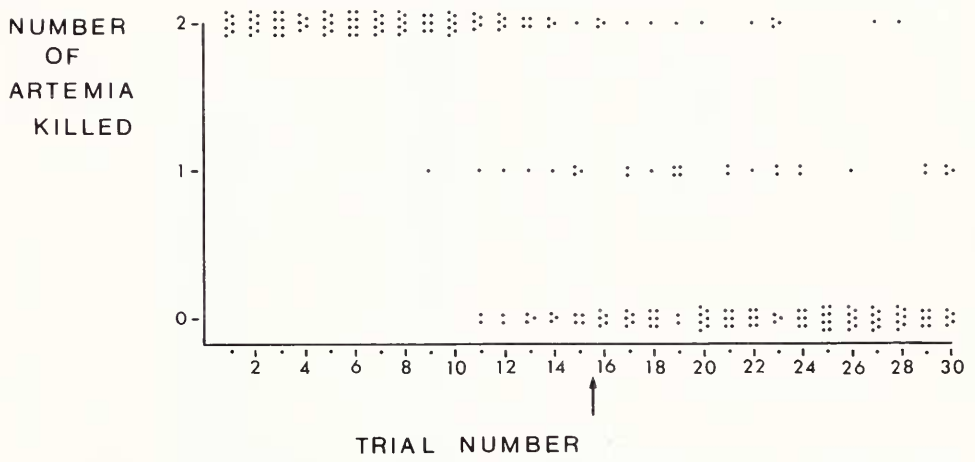

FIGURE 1. Number of brine shrimp killed/hydra when hydra were offered 2 brine shrimp at 5-min intervals for 30 trials. Arrow indicates the mean trial in which column contractions began. n = 8.

then kill one or two brine shrimp. Sometimes, brine shrimp that were captured were not killed. These would eventually pull free from the tentacles and continue swimming normally.

Application of brine shrimp homogenate (1/25 dilution) to the medium surrounding a hydra caused mouth opening and tentacle writhing responses. In these cases, when brine shrimp are placed near the tentacles of animals in homogenate, there is a significant decrease in the killing response compared to control animals in BVC solution ($P = .001$, Wilcoxon Rank Sums, Fig. 2). After a washing out period in which the hydra are placed in clean BVC solution, stenotele discharge returned and was (T_2, Fig. 2) significantly greater than that in the test animals (T_1, Fig. 2) ($P < 0.01$ Wilcoxon Signed-Rank).

Microscopic examination of hydra tentacles indicate that 1/25 dilutions of brine shrimp homogenates do not stimulate nematocysts to discharge. In both sets of experiments, test tentacles (dilute homogenate) were not significantly different from control tentacles (BVC solution). The five tentacles placed in homogenate for 5 min and covered with a coverslip produced no discharged nematocysts. Among control tentacles, one tentacle discharged a single stenotele. Among tentacles observed on an inverted microscope with no coverslip for 5 min, 3 out of 10 in homogenate discharged stenoteles (mean = 2.33 stenoteles/tentacle). Among 10 control tentacles, 4 discharged stenoteles (mean = 3.25 stenoteles/tentacle) and one discharged a desmoneme. No additional discharge was found at the 25 min interval. The results indicate that homogenate alone does not induce nematocyst discharge.

Almost complete inhibition appears to occur within 30 s of placing a hydra in diluted homogenate. No significant differences were found among the 5 tested time periods (30 s, 1, 2, 3, and 4 min.), indicating that the onset for inhibition was less than 30 s. Release from inhibition occurred in 20 min, after which the killing was restored to normal levels (Fig. 3).

In serial dilutions of homogenate, a linear relationship ($r = 0.82$) was found between the concentration of homogenate and the number of brine shrimp killed (Fig. 4). Although the number of brine shrimp killed by individual hydra varied, as a group

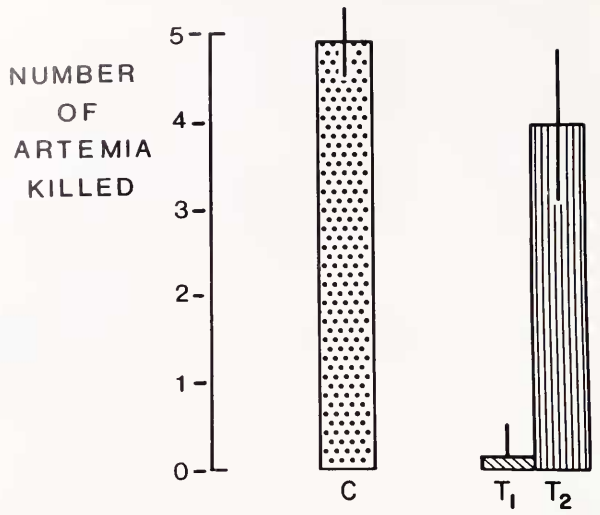

FIGURE 2. Number of brine shrimp killed (mean ± SD) during external application of 1/25 dilutions of homogenate. Each hydra was tested with five brine shrimp. C = Controls, in BVC solution; T_1 = tests, in dilute homogenate; T_2 = retests, retested after 45 min in BVC solution. n = 6.

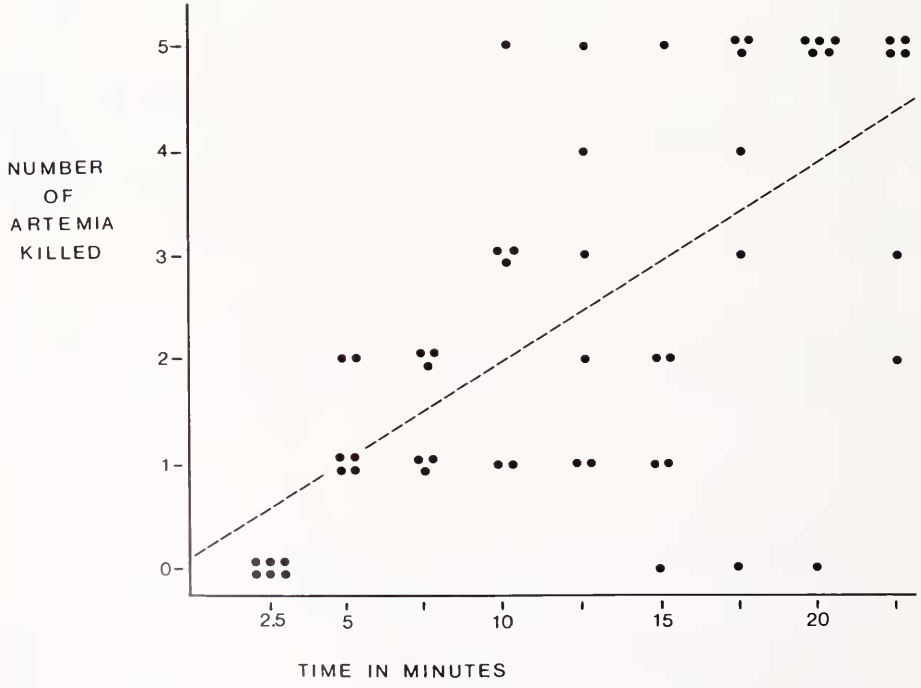

FIGURE 3. Release of stenotele nematocysts from inhibition. Hydra were placed in 1/25 dilutions of homogenate for 5 min and then transfered to fresh BVC solution. They were tested with five brine shrimp at given time intervals. n = 8, r = 0.67.

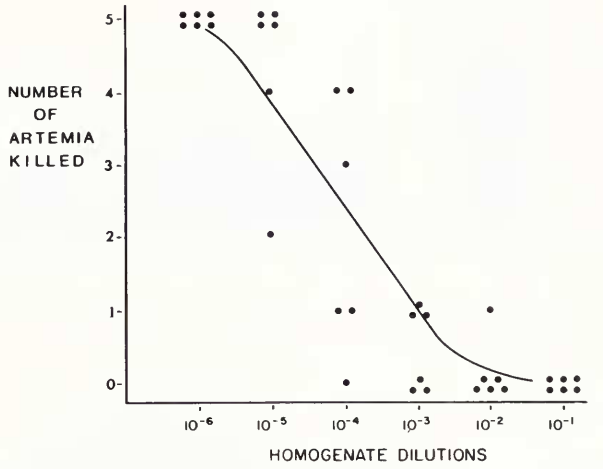

FIGURE 4. Serial dilutions of homogenate (18 mg/ml) that induce inhibition of stenotele discharge. Hydra were placed in given dilutions of homogenates for 5 min and then tested with 5 brine shrimp. n = 6. A linear regression was calculated between 10^{-5} and 10^{-2}. r = 0.82.

there was a 50% reduction in killing at a 10^{-4} dilution of homogenate. The protein concentration of the undiluted homogenate was 18 mg/ml. At 50% inhibition the protein concentration of the diluted sample was 1.8 μg/ml.

Brine shrimp homogenates separated according to molecular weight were tested for their effects on stenotele discharge and for various aspects of the feeding response (Fig. 5). Significant differences were found between the killing of brine shrimp in different fractions ($P = .0001$, Kruskal-Wallis). The differences were between the first two fractions (control) and the largest molecular weight fractions, 3 and 4 (Multiple Comparisons based on Kruskal-Wallis). The other samples did not show a significant loss in stenotele discharge compared to the controls.

There were also significant differences in the mouth opening response between the different fractions ($P = .0001$, Kruskal-Wallis). Fractions 8 through 11 had a significantly higher mouth opening response. Fractions 3 and 4 (the large mol. wt. fractions) caused little or no response. Significant differences were also found for tentacle activity ($P = .0001$, Kruskal-Wallis). Fractions 8 through 12 caused significantly more activity than the other samples.

GSH (5×10^{-4} M) was also run through the column. The results indicate that GSH had no effect on brine shrimp killing. Samples 7 through 11 produced at least ½ maximal mouth opening response (Table I). Tentacle activity responses were at least ½ maximal in samples 6 through 11 (Fig. 5). Little or no activity was found in samples 3 and 4 for either mouth opening response or tentacle activity.

Injections of whole homogenate into the gastrovascular cavity had no effect on stenotele discharge (Fig. 6). One hundred percent of the brine shrimp were killed during the seven testing periods. There was no indication of mouth opening or tentacle activity.

Only when homogenate leaked out of the hypostome (Fig. 6, T_2) was there a significant inhibition of stenotele discharge compared to control animals ($P = .009$, Wilcoxon Rank Sums). Leakage of homogenate can be caused by injecting excess amounts into the gastrovascular cavity and can be readily observed as a murky cloud

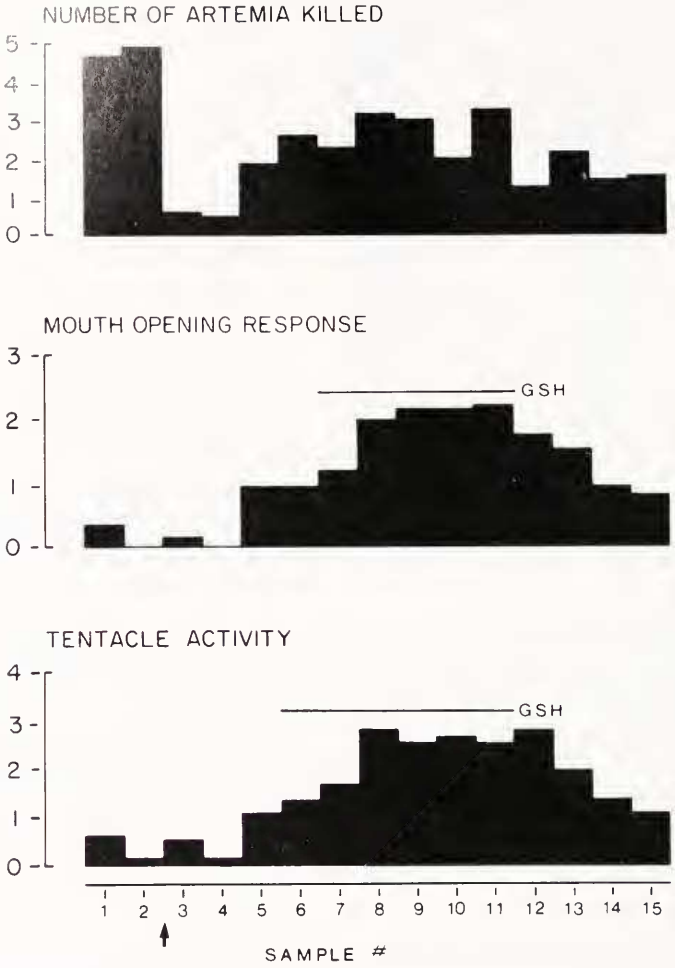

FIGURE 5. Homogenate separation using gel filtration chromatography. Values in each scale are defined in the methods section. The GSH lines indicate the samples with at least ½ maximal response to 5×10^{-4} reduced glutathione. Arrow indicates the beginning of the fractionated homogenate in the samples. n = 7.

around the tentacles. When this occurred, mouth opening responses and tentacle concerts or writhing were also observed.

Neither columns nor hypostomes are required for stenotele discharge to be inhibited (Fig. 7). Both ablated hypostomes with tentacles and isolated tentacles displayed significant inhibition of stenotele discharge compared to controls ($P = .001$, both experiments, Wilcoxon Rank Sums). Similar results were found by Smith *et al.* (1974) and Ruch and Cook (1984) using hypostomes and tentacles.

Treatment of the base of tentacles with homogenate (Fig. 8, T_1) resulted in a significant loss of stenotele discharge compared to the tentacle base of control animals ($P = .003$, Wilcoxon Rank Sums). Brine shrimp killing for the tips of the tentacles, which had not been treated, were not significantly different from their corresponding controls. After placing the base of the tentacles in fresh BVC solution for 1 h, stenotele

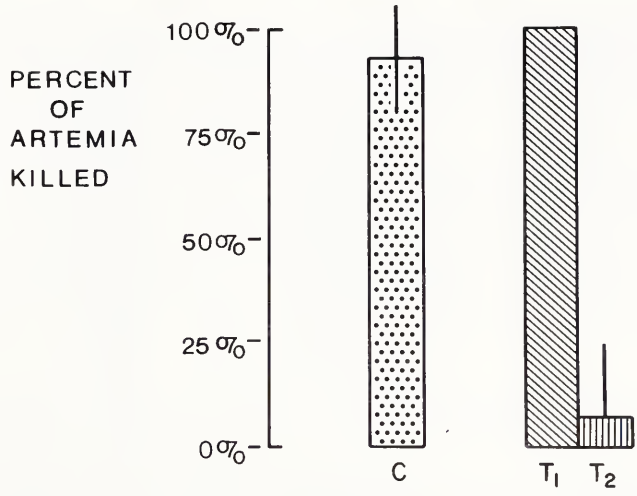

FIGURE 6. Injections of homogenate into the gastrovascular cavity through the basal pore. Control animals were injected with air. Results expressed as % brine shrimp killed (mean ± SD) by the tentacles. Percents were used since the total number of brine shrimp used varied in each test (T_1 = 14 brine shrimp, T_2 = 5). C = Controls, air injections; T_1 = test, homogenate injections; T_2 = retests, homogenate leaking out of the gastrovascular cavity. n = 5.

discharge in the base of the tentacles (Fig. 8, T_2) largely returned and was significantly different from the base of the test tentacles ($P < 0.01$ Wilcoxon Signed-Rank).

DISCUSSION

Stenotele discharge in hydra becomes inhibited when animals are fed to repletion. After repletion and the onset of inhibition, the animals remain at least partially inhibited until the end of the experiment (95 min). This compares favorably with results by Smith *et al.* (1974) which indicate that stenotele discharge remains partially inhibited until regurgitation (over 4 h).

Externally applied brine shrimp homogenates produced a rapid onset of inhibition of stenotele discharge (<30 s). Since the methods used to measure the time of onset were not sensitive enough to resolve times less than 30 s, a more exact time for onset of inhibition could not be determined. The release from inhibition is relatively slow. These findings also agree with work by Smith *et al.*, (1974) in which the hypostome and tentacles were removed from inhibited animals and placed in fresh culture solution; the killing response returned in 30 min.

The inhibitory effects of the homogenates were dependent upon concentration. At 50% inhibition of stenotele discharge, the concentration of homogenate was equal to the water soluble layer of 0.043 brine shrimp/μl (1.8 μg/ml protein). This is a very small amount compared to the average number ingested by experimental animals (mean = 32 brine shrimp/hydra). Although the number of shrimp necessary to induce 50% inhibition of stenotele discharge is greater than the average number ingested by a hydra, it is probably incorrect to calculate the concentration of the inhibitory substances, surrounding a repleted and inhibited hydra, as though they were dissolved in the entire experimental dish. Loomis (1964) and Lenhoff (1965) showed that a concentration gradient does exist in the microenvironment surrounding a hy-

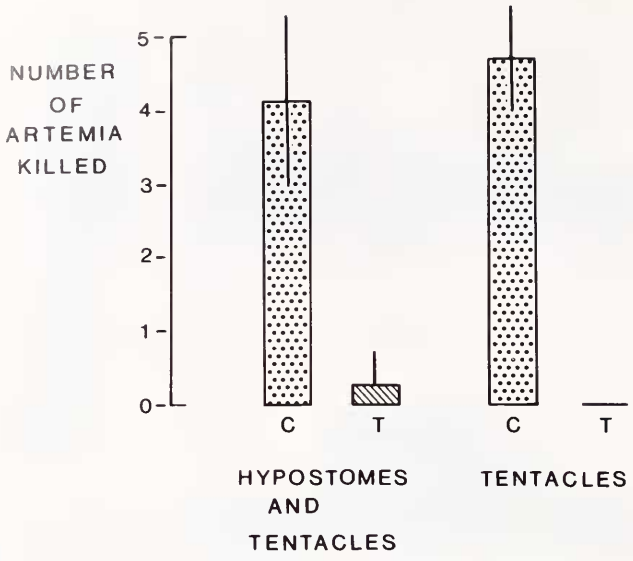

FIGURE 7. Inhibition of stenotele discharge in hypostomes and tentacles of hydra. Ablated hypostomes with tentacles (n = 8) and isolated tentacles (n = 9) were placed in 1/25 dilutions of homogenate for 5 minutes and tested with 5 brine shrimp. C = controls, in BVC solution; T = tests, in dilute homogenate. Bars represent standard deviations.

dra which can have behavioral effects. The effective concentration of metabolites, surrounding a hydra, can not be extrapolated from our data.

Two responses are elicited when homogenates are applied to the medium sur-

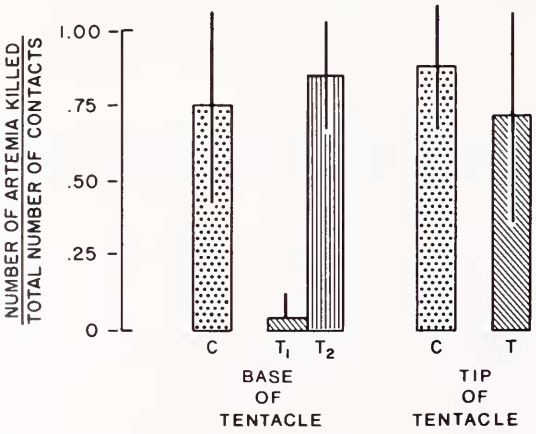

FIGURE 8. Bases and tips of intact tentacles were tested for inhibition of stenotele discharge in 1/25 dilutions of homogenate. The base of the tentacle was covered with homogenate for 2 min and then the entire tentacle was tested with excess brine shrimp for 2 min. Inhibited regions of the tentacle were ablated, placed in fresh BVC solution, and retested after 1 h. The scale represents the ratio of the number of brine shrimp killed over the total number of contacts (mean ± SD). C = controls, tentacle placed in BVC solution only; T_1 = test, base of tentacle placed in homogenate; T_2 = retest, base of tentacle after washing for 1 h in BVC solution. n = 8.

rounding a hydra: (1) an activation of the feeding reflex (mouth opening and tentacle movement) and (2) an inhibition of stenotele discharge. The strong activation of the feeding reflex does not cause the decrease in the killing of brine shrimp because there is no decrease in killing when GSH is applied to the external medium (GSH causes a strong activation of the feeding reflex).

Ruch and Cook (1984) proposed that inhibition of stenotele discharge was due to the previous discharge of nematocysts. They found that instead of inhibition, brine shrimp homogenates (5.8 μg/1.5 ml) caused a 35% increase in stenotele discharge. Only when they applied a "crude homogenate" (of an unknown concentration) were they able to get a significant decrease in stenotele discharge. They attributed this inhibition to the large number of stenotele nematocysts that were discharged by the homogenate itself. According to their data 75 to 100 nematocysts were discharged per hydra.

We are unable to confirm these findings with our experiments. In our experiments, homogenates diluted to a similar protein concentration (1.8 μg/ml) induced 50% inhibition of stenotele discharge. The inhibition of stenotele discharge is not due to previous nematocyst discharge, since at higher protein concentrations (0.74 mg/ml), where there is complete inhibition of stenotele discharge, no significant amount of nematocyst discharge occurred. Our work is consistant with that of Pantin (1942) and Ewer (1947) who found that food extract alone does not elicit nematocyst discharge, although it does lower the threshold to discharge when applied locally. Since homogenate is the only variable altered in our experimental design, we believe that a substance in the homogenate causes the inhibition of stenotele nematocysts.

Ruch and Cook (1984) showed that nematocyst-rich tissues will cause complete inhibition of stenotele discharge. Furthermore, Clark and Cook (1986), in the colonial hydroid *Halocordyle disticha,* were able to induce complete inhibition using the discharge products of large numbers of purified stenotele nematocysts. It is possible that more than one mechanism for inhibition of stenotele discharge exists or that the various mechanisms may employ a similar substrate.

Gel filtration chromatography demonstrates that the substances activating the feeding reflex and those inhibiting stenotele discharge do not have the same molecular weight. The largest molecular weight fraction (>5000), which caused the strongest inhibition of stenotele discharge, displayed very little or no activation of the feeding reflex, whereas the samples causing the strongest feeding reflex also produced the highest level of killing. Since gel filtration chromatography separates substances based upon molecular weight, the partial inhibition in later column samples indicates either that inhibition of stenotele discharge may be caused by more than one substance or that there was incomplete separation of the homogenate samples.

Injections of brine shrimp homogenates into the basal disc of hydra do not cause any inhibition of stenotele discharge or activation of the feeding response until homogenate leaks into the external environment. Furthermore, different body regions or even different regions of a tentacle behave independently from each other. Whether inhibition occurs directly at the nematocyte or involves some other component of the battery cell complex (Hufnagel *et al.,* 1985) remains to be determined.

In summary, inhibition of stenotele discharge can be induced with external application of prey homogenates. The onset of inhibition is rapid (<30 s), while the release from inhibition is relatively slow (>20 min). Inhibition is dependent upon the concentration of homogenate. The inhibitory substances are different from those which activate the feeding reflex. The receptors for these inhibitory substances are found on the external surface of the hydra tentacle and may be associated with the nematocyte directly or with some other component of the battery cell complex.

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